Exhibit 4

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RAPID COMMUNICATIONS

Inhibition of Stress-Activated MAP Kinases Induces Clinical Improvement in Moderate to Severe Crohn's Disease

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Background & Aims: We investigated if inhibition of mitogen-activated protein kinases (MAPKs) was beneficial in Crohn's disease. *Methods:* Inhibition of JNK and p38 MAPK activation with CNI-1493, a guanylhydrazone, was tested in vitro. Twelve patients with severe Crohn's disease (mean baseline, CDAI 380) were randomly assigned to receive either 8 or 25 mg/m² CNI-1493 daily for 12 days. Clinical endpoints included safety, Crohn's Disease Activity Index (CDAI), Inflammatory Bowel Disease Questionnaire, and the Crohn's Disease Endoscopic Index of Severity. *Results:* Colonic biopsies displayed enhanced JNK and p38 MAPK activation. CNI-1493 inhibition of both JNK and p38 phosphorylation was observed in vitro. Treatment resulted in diminished JNK phosphorylation and tumor necrosis factor production as well as significant clinical benefit and rapid endoscopic ulcer healing. No serious adverse events were noted. A CDAI decrease of 120 at week 4 ($P = 0.005$) and 146.5 at week 8 ($P = 0.005$) was observed. A clinical response was seen in 67% of patients at 4 weeks and 58% at 8 weeks. Clinical remission was observed in 25% of patients at week 4 and 42% at week 8. Endoscopic improvement occurred in all but 1 patient. Response was seen in 3 of 6 infliximab failures, 2 of whom showed remission. Fistulae healing occurred in 4 of 5 patients, and steroids were tapered in 89% of patients. *Conclusions:* Inflammatory MAPKs are critically involved in the pathogenesis of Crohn's disease and their inhibition provides a novel therapeutic strategy.

Crohn's disease (CD) is a chronic inflammatory disorder of the gastrointestinal tract, which is thought to arise in genetically susceptible hosts caused by an inappropriate immunologic response against the microflora of the gut. The immune defect is unclear; however, recently a gene for CD was identified encoding NOD2, a protein involved in the recognition of microbes and signalling events leading to an immune response.[1,2] This finding directly links the mucosal immune response to enteric bacteria and the development of disease.

Tumor necrosis factor (TNF) plays a central role in the initiation and amplification of the inflammatory reaction observed in CD.[3] Monoclonal antibodies against TNF have been proven efficacious in both inducing clinical remission and endoscopic healing.[4,5] An alternative means of inhibiting TNF action is by inhibition of mitogen-activated protein kinases (MAPKs), signal-transducing enzymes that regulate important cellular processes like gene expression and cell proliferation.[6] Targeting the p38 MAPK signalling cascade led to reduction of lipopolysaccharide (LPS)-induced TNF production in rodents.[7] Hence, MAPK inhibition has been suggested as an anti-inflammatory strategy.[8] However, evidence that these proteins are required for the pathogenesis of inflammatory disease and that MAPK inhibition constitutes a therapeutic target is lacking.

An interesting candidate would be CNI-1493, a guanylhydrazone that inhibits the phosphorylation of both p38 MAP kinase and JNK.[8,9] CNI-1493 has been shown to inhibit macrophage activation and the production of several proinflammatory cytokines (TNF-α, IL-1, IL-6, MIP-1α, MIP-1β) and nitric oxide.[10,11] Furthermore, it was shown to be protective in a number of preclinical models, including endotoxic shock,[10,12] acute respiratory distress syndrome,[13,14] sepsis,[10] pancreatitis,[13,14] experimental allergic encephalomyelitis,[15] stroke,[16] rheumatoid arthritis, and dextran sulfate sodium colitis (data on file).

Abbreviations used in this paper: CDEIS, Crohn's Disease Endoscopic Index of Severity; CRP, C-reactive protein; HRP, horseradish peroxidase; IBDQ, Inflammatory Bowel Disease Questionnaire; LPS, lipopolysacchride; MAPK, mitogen-activated protein kinase; PBMCs, peripheral blood mononuclear cell; TNF, tumor necrosis factor.

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The goal of the current study was to investigate if MAP kinase activation was present in patients with CD, and to examine if inhibition of MAP kinase activation was a safe and effective novel therapeutic approach.

Materials and Methods

Effect of CNI-1493 on JNK and p38 MAPK Activation In Vitro

Peripheral blood mononuclear cells (PBMCs) obtained from healthy volunteers were stimulated with LPS for 15 minutes in the presence of increasing concentrations of CNI-1493 or diluent. Analysis of MAPK phosphorylation was performed using Western blotting and phosphospecific antibodies (Cell Signalling, Beverly, MA).

Patients

Twelve patients were enrolled in a doubled-blinded fashion to receive either 8 or 25 mg/m^2 of CNI-1493 intravenously once daily for 12 consecutive days. Patients were required to suffer from moderate to severe CD, i.e., a Crohn's Disease Activity Index (CDAI)[17] of $\geq$ 220 and $\leq$ 450; to have a CD history of at least 3 months duration, with colitis, ileitis, or ileocolitis confirmed by radiography, endoscopy, and histology. Furthermore, patients had to be on a stable dose of corticosteroids, aminosalicylates, or antibiotics at least 4 weeks before inclusion. Patients receiving methotrexate, 6-mercaptopurine, or azathioprine should have had stable dosages for at least 8 weeks before inclusion. Patients with extensive bowel resection (e.g., >100 cm of small bowel, proctocolectomy, or colectomy with ileorectal anastomosis) or fixed stenosis were excluded.

The primary endpoints were safety evaluations, as determined by occurrence of (1) adverse events and (2) stopping medication because of adverse events. The secondary endpoints were efficacy evaluations: (1) occurrence of a clinical response as defined by achieving a reduction of CDAI of $\geq$25% and $\geq$70 points as compared with baseline, occurring at least once after the start of treatment, and (2) occurrence of a clinical remission, defined as a reduction of CDAI to below 150, occurring at least once after the start of treatment. Safety and tolerability was evaluated by clinical assessment of adverse events and changes in standard hematologic and biochemical laboratory parameters. Other clinical assessments of efficacy included the Inflammatory Bowel Disease Questionnaire (IBDQ),[18] the Crohn's Disease Endoscopic Index of Severity (CDEIS),[19] and C-reactive protein (CRP) levels. Increasing IBDQ scores indicate improvement, values above 170 are considered normal. The CDEIS is a score, which is based on the presence of deep or superficial ulceration, the proportion of ulcerated surface, and the presence of ulcerated or nonulcerated stenosis in the terminal ileum and 4 different segments of the colon. At every visit, enterocutaneous or rectovaginal fistulas were examined to determine whether a fistula was present, open or closed. An enterocutaneous fistula was considered to be closed when it was no longer draining despite gentle compression. Rectovaginal fistulas were considered to be closed, based on either physical examination or absence of relevant symptoms (passage of rectal material or flatus from vagina). Written informed consent has been obtained from all patients, and the study has been approved by the Ethical Committee of the Academic Medical Center, Amsterdam.

Immunohistochemistry

Mucosal biopsy specimens of the intestine were obtained during videoendoscopy at the time of screening and at the end of week 4 for 6 patients. At both occasions biopsies were taken from most affected regions of inflammation, and subsequently formalin fixed and paraffin embedded. If patients were in remission at week 4, biopsies were taken form areas with apparent residual inflammatory changes. As a control, histological normal biopsies were obtained from non-CD patients. Paraffin sections (4 μm) were dewaxed and rehydrated in graded alcohols. Endogenous peroxidase activity was quenched with 1.5% H_2O_2 in phosphate-buffered saline (PBS) for 30 minutes at room temperature. Nonspecific staining was blocked with 10 mmol/L Tris, 5 mmol/L EDTA, 0.15 mol/L NaCl, 0.25% gelatin, 0.05% (vol/vol) Tween 20, pH 8.0, for 30 minutes at room temperature. After a washing with PBS the after primary Abs were applied: a mouse monoclonal anti-human antibody to phosphorylated JNK (Santa Cruz, CA; 1:400), a mouse monoclonal anti-human antibody to phosphorylated p38 MAPK (Cell Signalling, Beverly, MA; 1:25) or a mouse monoclonal immunoglobulin (Ig) M Ab against TNF-α, clone 4C6-H6 (Instruchemie, Hilversum, the Netherlands; 1:25). Sections were stored overnight at 4°C. The following day sections were washed in PBS and incubated with a secondary biotinylated goat anti-mouse Ig Ab (DAKO, Glostrup, Denmark; 1:200) for 1 hour at room temperature and washed with PBS. Detection was performed either with horseradish peroxidase (HRP) as an enzyme. Sections were incubated with HRP conjugated ABcomplex (DAKO) for 60 minutes and peroxidase activity was detected with diaminobenzidine (fast DAB, Sigma, St. Louis, MO). Sections were briefly counterstained with hematoxylin when appropriate, dehydrated in graded alcohols, and mounted. Controls consisted of omitting the primary and secondary Ab and use of an appropriate Ig control.

MAPKs Activation and TNF Immunohistochemistry

To assess the amount of TNF and the activity of JNK and p38 in situ in the human colon, screening and week 4 specimens available from 6 patients and controls were stained for TNF-α and phosphorylated JNK/p38. No counterstaining was applied to these sections to visualize positive cell more clearly. Three pictures of each section were taken at ×400 magnification, and positive cells were counted, blind to treatment and day of endoscopy, in each microscope field with the use of an image analysis program (EFM Software, Rotterdam, the Netherlands). Pictures appeared randomly on a computer

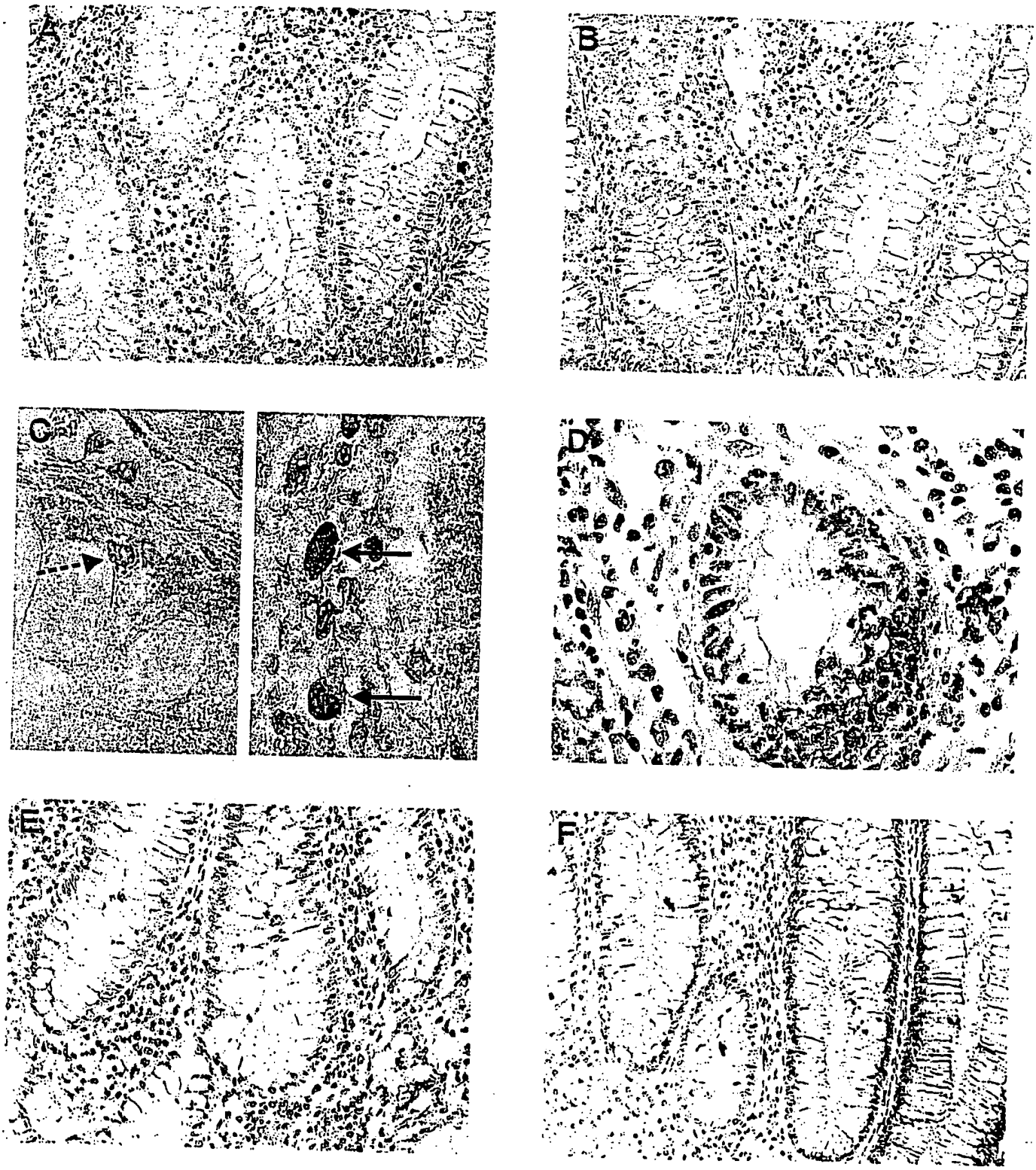

Figure 1. Involvement of MAPKs in CD. Patients received 12 days of intravenous infusions with CNI-1493 (8 or 25 mg/m^2). Paired biopsies were obtained at screening (day 1) and at week 4 for 6 patients. Sections were stained for phospho-JNK, phospho-p38 MAPK, and TNF-α. (*A*) Many inflammatory cells stained positive for phospho-JNK (original magnification, 200×). (*B*) After treatment with the MAPK inhibitor, a reduced number of cells stain positive for phospho-JNK (original magnification, 200×). (*C*) Inflammatory cell staining positive for phospho-JNK were IELs (*dotted arrow*, CD3+) or LP macrophages (*closed arrows*, CD68+) (1000×). (*D*) Activated p38 MAPK was observed in migrating neutrophils localized in crypt abscesses (1000×). The number of TNF staining cells in the lamina propria in biopsy specimens taken (*E*) before CNI-1493 treatment (400×) was significantly higher than in specimens taken (*F*) after treatment (400×).

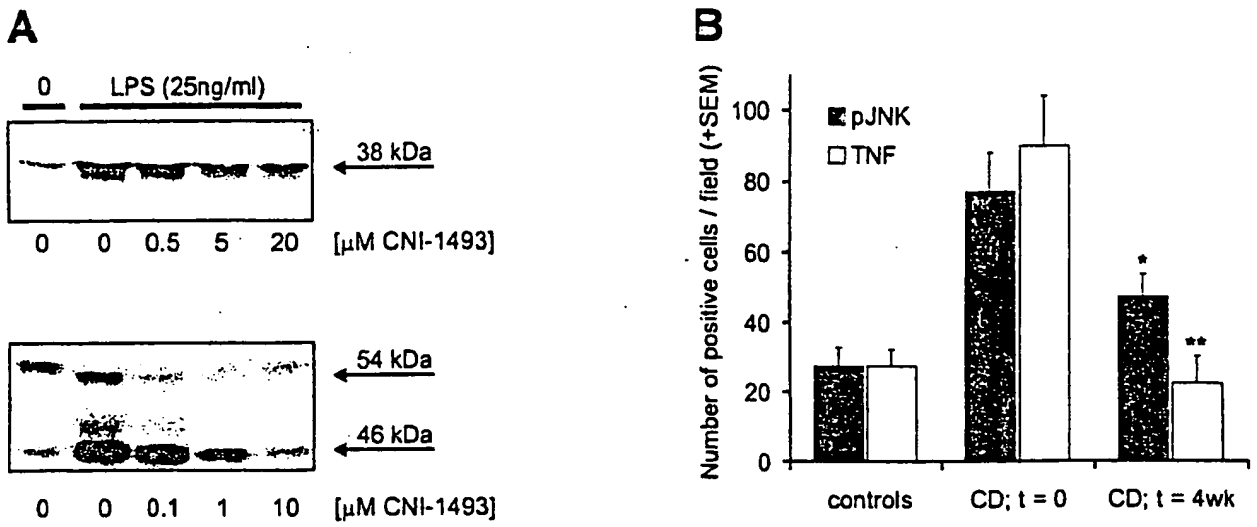

Figure 2. Effect of CNI-1493 in vitro and in CD. (*A*) Effect of CNI-1493 on LPS-induced JNK and p38 MAPK phosphorylation in PBMCs. Cells were stimulated with LPS (25 ng/mL) for 15 minutes or solvent control (0) in the presence of various concentrations CNI-1493. JNK and p38 MAPK activation was assessed with Western blotting using antibodies against phosphorylated JNK (against both 46- and 54-kilodalton isoforms, 1:1000) and p38 MAPK (38 kilodalton, 1:1000). (*B*) CNI-1493 inhibits the number of phospho-JNK and TNF-positive cells in CD. Patients received 12 days of intravenous infusions with CNI-1493 (8 or 25 mg/m^2). Paired biopsies were obtained at screening (day 1) and at week 4 for 6 patients. Sections were stained for phospho-JNK or TNF-α without applying hematoxylin counterstain. Three pictures of each section were taken at original magnification 400×, and positive cells were counted, blind to treatment and day of endoscopy, in each microscopic field with the use of an image analysis program. As a control, 8 histological normal biopsies were obtained from non-CD patients, and were counted in the same fashion. Differences between pretreatment and posttreatment reached statistical significance (JNK, $*P = 0.01$; TNF, $**P < 0.01$).

monitor and all intensely staining cells were marked positive by an observer, counted, and stored by the image analysis program for later data analysis.

Statistical Analysis

All measures of efficacy were evaluated by intention-to-treat analysis. Change from baseline within groups was analyzed by use of the Wilcoxon signed rank test. Change from baseline between groups at a specified time point was analyzed by use of the Wilcoxon rank-sum test. Comparisons between groups over the course of study were evaluated by use of repeated measures analysis of variance. Last observation carried forward analysis was implemented for missing data. The correlation between CDAI and CDEIS change from baseline was analyzed by use of the Pearson correlation coefficient. Baseline categorical variables were analyzed with Fisher exact test and continuous variables with the Wilcoxon rank-sum test.

Results

JNK and p38 MAPK Are Activated in Active CD

Although MAPKs have been implicated in regulation of inflammatory responses, actual involvement of MAPKs in chronic inflammatory disease has not yet been demonstrated. Recently, Waetzig et al.[20] reported increased activity of stress-activated MAPKs in CD. In colonic biopsies taken from patients with active CD, we show that activation of JNK and p38 MAPK is markedly present (Figure 1*A* and *D*). Activated p38 MAPK was observed in infiltrating neutrophils (Figure 1*D*) and in epithelial cells (not shown). Abundant active JNK was observed in inflammatory cells, especially intraepithelial lymphocytes (IELs, CD3$^+$) and lamina propria cells ([LPs] macrophages, CD68$^+$) stained positive (Figure 1*A* and *C*). Quantative comparison to histological normal biopsies showed that the number of phopspho-JNK positive cells was significantly increased (Figure 2*B*). As expected, the number of TNF-α positive cells in the colon mucosa of CD patients was also significantly increased compared with normal controls (Figure 2*B*).

CNI-1493 Is a Potent JNK and p38 MAPK Inhibitor In Vitro

CNI-1493 is reported to inhibit stress-activated MAP kinases in lymphocyte cell lines.[21] To confirm whether this compound exerts similar action in untransformed cells, we stimulated PBMCs with LPS in the presence of increasing concentrations of CNI-1493. CNI-1493 inhibited LPS-induced phosphorylation of both p38 MAPK and JNK in a dose-dependent fashion, although apparently with a higher efficacy for inhibiting JNK (Figure 2*A*). Thus, CNI-1493 effectively inhibits the stress-activated MAPKs in untransformed cells. Therefore, we decided to use this inhibitor for elucidating their role in CD.

Safety and Efficacy of CNI-1493 in CD

We included 12 patients with severe CD (Table 1), 8 completed therapy. Two discontinued (after 6 and 9 doses of medication, both receiving 25 mg/m^2 daily) because of elevated alanine aminotransferase levels; one after 11 doses caused by a catheter-related infection (8 mg/m^2 daily), and one after 9 doses because of deterioration of CD (25 mg/m^2 daily). Treatment was generally well tolerated, side effects included phlebitis in 2 patients in each dosing group, and asymptomatic and transient elevation of liver enzymes in 1 patient in the low-dose and 5 patients in the high-dose group (Table 2).

For both dose groups combined, we observed a median change from baseline CDAI of −117.5 at 2 weeks (P = 0.003), −120 at 4 weeks (P = 0.005), −146.5 at 8 weeks (P = 0.005), and −148 at 16 weeks (P = 0.007). Figure 3*A* shows CDAI scores, data are censored at the last observation before any change in concomitant Crohn's therapy or addition of other medication, and the last observation is carried forward. Table 3 summarizes the response rates as well as the remission rates according to the predefined criteria. At 4 weeks from the start of treatment, 25% of patients were in remission, and 67% had responded. At 4 months after the start of treatment, without additional medications, half the patients were in remission and 58% were responders. The response rates in both treatment groups were similar, though the small sample size of each group precludes precise conclusions. Three of 6 patients who did not respond to prior infliximab therapy showed a response after CNI-1493 administration, of whom 2 entered remission. In parallel with the CDAI changes, we observed a median IBDQ increase from baseline of 21.5 at 2 weeks (P = 0.02), 36.5 at 4 weeks (P = 0.01), 43 at 8 weeks (P = 0.007), and 33.5 at 16 weeks (P = 0.002) in both groups combined (Figure 3*B*). CRP levels decreased significantly during the first weeks of treatment (Figure 3*C*). At week 4, 16 days after the end of therapy, endoscopic improvement was observed in all but 1 patient (Figure 3*C* and *D*), with a median change in CDEIS from baseline of −6.5 (P = 0.006).

Five patients suffered from active fistulizing CD, and closure of fistulae was observed in 4 patients during the study period (1 in the 8 mg/m^2 and 3 in the 25 mg/m^2 group). At baseline, 9 patients were receiving steroids, 5 used prednisolone (mean, 21 mg; range, 10–40 mg), and 4 used budesonide (all 9 mg). At week 8, steroids had been tapered in 8 patients (89%). In patients receiving prednisolone, the mean reduction was 12.5 mg at week 8; in the budesonide group, the mean reduction was 5 mg. CD-related arthralgia/arthritis was reported in 5 of 12 study patients at baseline, but resolved in all during the study period. We concluded that a 12-day infusion with CNI-1493 was safe and induces significant endoscopic healing and substantial clinical benefit in moderate to severe CD.

Table 1. Patient Characteristics

	8 mg/m^2	25 mg/m^2
Gender (M/F)	0/6	2/4
Age (yr)[a]	27.5 (19.8–35.6)	44.7 (18.7–54.4)
Duration of disease (yr)	5.7 (0.1–12.0)	13.8 (8.8–31.6)
CDAI[b]	382.5 (288–507)	377.5 (284–440)
IBDQ[c]	103 (50–149)	82.5 (79–132)
CDEIS[d]	14.4 (7.6–25)	12.2 (1.7–24.3)
Prior intestinal resection (N)	1	5
Fistulae	2/6	3/6
Concomitant medication[e]		
Mesalamine	1	3
Corticosteroids	4	5
Azathioprine	4	4
Prior Infliximab (N)	3	3

[a]Values expressed as median (range); [b]CDAI; [c]IBDQ; [d]CDEIS; [e]Concomitant Crohn's disease therapy.

Table 2. Adverse Events

Body system/ adverse event	Daily dose, mg/m^2 8	25	Body system/ adverse event	Daily dose, mg/m^2 8	25
N=	6	6	Any AE possibly/ probably related	6	5
Body general	2	3	Digestive/hepatic	3	5
Abdominal pain	0	1	Alk phos increase	0	3
Asthenia	1	0	Bilirubinemia	1	0
Facial edema	0	1	Nausea	1	1
Flu syndrome	1	1	SGOT increase	1	5
Headache	1	1	SGPT increase	0	5
Cardiovascular	2	2	Stomatitis	0	1
Hematoma	1	0	Nervous	1	0
Phlebitis	2	2	Dizziness	1	0
Hematologic	4	2	Respiratory	1	1
Anemia	2	2	Rhinitis	0	1
Leukopenia	1	1	Skin	2	0
Thrombocytopenia	1	0	Herpes Zoster	1	0
			Maculopapular rash	1	0

CNI-1493 Inhibits JNK Phosphorylation In Vivo

To establish the effects of CNI-1493 treatment on MAPKs in vivo, paired biopsies (available from 6 patients), taken before (day 1) and after (week 4) treatment with CNI-1493 were stained for phospho-JNK and -p38 MAPK. The phospho-p38 MAPK staining was not consistent throughout all biopsies and thus, based on our immunohistochemistry, no assessment could be made of the effectiveness of CNI-1493 in inhibiting p38 MAPK

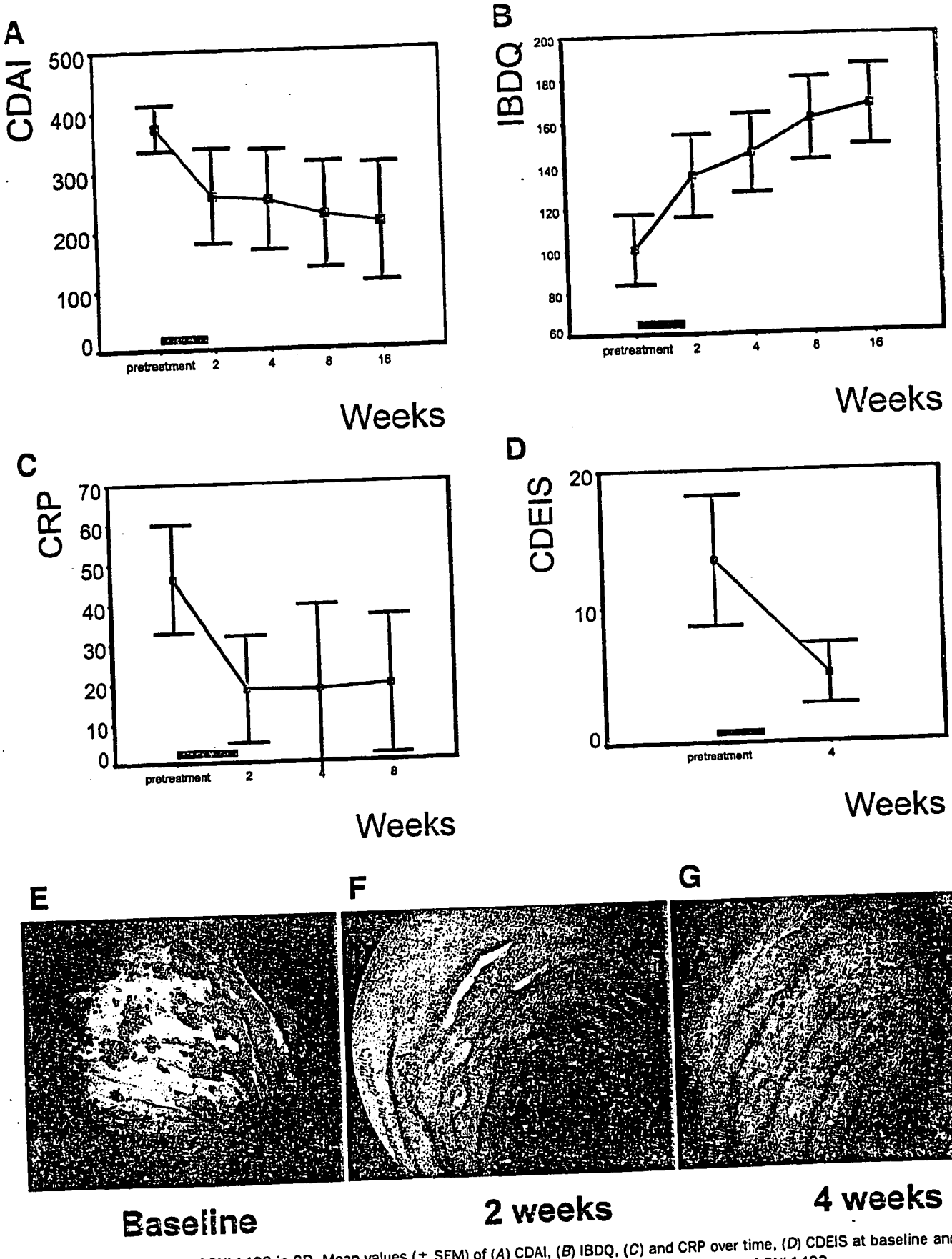

Figure 3. Clinical effect of CNI-1493 in CD. Mean values (± SEM) of (*A*) CDAI, (*B*) IBDQ, (*C*) and CRP over time, (*D*) CDEIS at baseline and da 29; (*E–G*) example of the endoscopic findings at baseline, 2 and 4 weeks after 25 mg/m^2 administration of CNI-1493.

Table 3. Number of Patients Showing a Response or Remission After Treatment With CNI-1493

Dose group (mg/m^2)		Study day				
		8	15	29	57	112
		Number (%) in remission/responding				
8	Remission[a]	0	0	1 (17)	2 (33)	3 (50)
	Response[b]	2 (33)	5 (83)	5 (83)	4 (66)	4 (66)
25	Remission	0	3 (50)	2 (33)	3 (50)	3 (50)
	Response	3 (50)	3 (50)	3 (50)	3 (50)	3 (50)
Overall	Remission	0	3 (25)	3 (25)	5 (42)	6 (50)
	Response	5 (42)	8 (67)	8 (67)	7 (58)	7 (58)

[a]Occurrence of a clinical remission, defined as a reduction of CDAI to below 150, occurring at least once after the start of treatment.
[b]Occurrence of a clinical response as defined by achieving a reduction of CDAI of ≥25% and ≥70 points as compared with baseline, occurring at least once after the start of treatment.

in vivo. In contrast, treatment with CNI-1493 decreased the number of cells staining positive for active JNK (Figure 1*B*). Quantitatively confirmation came from experiments in which the number of phospho-JNK positive cells were counted in sections that were not counterstained with hematoxylin (in a blinded fashion). The MAPK inhibitor treatment strikingly reduced the number of phospho-JNK positive cells in these biopsies ($P = 0.01$, Figure 2*B*). We concluded that the clinical benefit of CNI-1493 coincides with reduction of active JNK.

Effects on Local TNF-α Production

The effectiveness of CNI-1493 treatment was further confirmed in patients and controls by staining for TNF-α, a proinflammatory cytokine that was reported to be under the regulatory control of JNK and p38 MAPK. Many TNF-α positive cells were found in the lamina propria (Figure 1*E*). After treatment with CNI-1493 the number of TNF-α positive cells in the colon mucosa significantly decreased, compared with the biopsies taken at time of screening (Figure 1*F*).

Discussion

We tested CNI-1493, a synthetic guanylhydrazone known to inhibit both JNK and p38 MAPK, in patients with moderate to severe CD. Although it is now generally recognized that inflammation involves the activation of stress-activated MAPKs, the actual importance of these kinases in human pathology is poorly understood. CNI-1493 has been shown to be protective in several experimental models involving inflammatory cytokine excess; however, clinical experience with CNI-1493 is limited. In a phase I study, CNI-1493 was studied in melanoma and renal cancer patients receiving high-dose IL-2.[22] CNI-1493 was well tolerated and inhibited the IL-2-induced increase in TNF-α in a dose-dependent fashion. A pilot study of CNI-1493 in patients with moderate to severe psoriasis showed a marked response in several patients to a brief course of therapy, which lasted for up to several months without further therapy (data on file).

In this pilot study, we show that CNI-1493 has significant therapeutic impact on severe CD, resulting in endoscopic healing, as well as remarkable and sometimes long-lasting clinical benefit. Concomitantly, the strong JNK phosphorylation observed in patients before the onset of treatment was reduced, implying that CNI-1493 treatment caused JNK inhibition in vivo. These results support the notion that inflammatory MAPKs are critically involved in the pathogenesis of CD.

Immunohistochemical staining of colon biopsies for p38 MAPK did not yield consistent results, nor was a decrease in phosphorylated p38 MAPK staining detected after CNI-1493 treatment. In agreement, directly determining p38 MAPK activity, using immunoprecipated p38 MAPK from colon biopsies in in vitro kinase assays (not shown), did not reveal an influence of CNI-1493 treatment on p38 MAPK enzymatic activity. Together with our data showing that CNI-1493 more potently inhibits JNK phosphorylation in LPS-stimulated PBMCs in vitro and in mucosal inflammatory cells in vivo, our studies suggest that JNK is the more relevant target for CNI-1493 treatment. These findings would correspond well with recent results obtained in our laboratory obtained with TNBS-induced colitis in mice, which revealed that although the p38 MAPK inhibitor SB20358 effectively inhibited p38 MAPK enzymatic activity in these mice, no attenuation of disease progression was observed.[23] Hence, we favor the hypothesis that JNK inhibition underlies the clinical benefit of CNI-1493 in CD, but until more JNK-specific inhibitors are tested other possibilities must be kept in mind.

To our knowledge, this is the first article that reports immunocompetent cells within the inflamed human intestinal lamina propria expressing phosphorylated JNK and p38 MAPK. Whatever the exact nature of these underlying inflammatory MAPKs, the present study indicates that activity of these kinases is essential for CD-pathogenesis. In this open-label pilot study we observe that inhibition of these kinases may have significant clinical benefit, resulting even in endoscopic healing. As inhibition of such kinases may be achieved with small, orally available, and relatively cheap compounds we propose that such a therapy may constitute a promising novel avenue for the treatment of CD.

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